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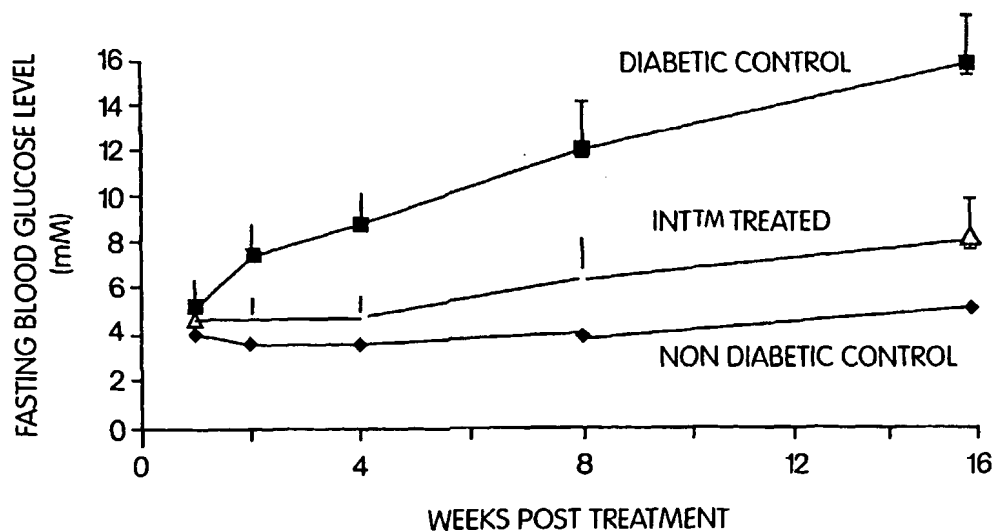
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(54) Title: PROLONGED EFFICACY OF ISLET NEOGENESIS THERAPY METHODS WITH A GASTRIN/CCK RECEPTOR LIGAND AND AN EGF RECEPTOR LIGAND COMPOSITION IN SUBJECTS WITH PREEXISTING DIABETES



(57) Abstract: Compositions and methods are provided for achieving in vivo islet cell regeneration in subjects with preexisting diabetes. The methods comprise short term treatment with a composition having a gastrin/cholecystokinin receptor ligand and an EGF receptor ligand. Treatment with such a composition for a short term resulted in a prolonged period of increased insulin release, decreased fasting blood glucose, and improved glucose tolerance, the prolonged efficacy, the period being considered from the time of cessation of treatment.



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PROLONGED EFFICACY OF ISLET NEOGENESIS THERAPY METHODS WITH A
GASTRIN/CCK RECEPTOR LIGAND AND AN EGF RECEPTOR LIGAND
COMPOSITION IN SUBJECTS WITH PREEXISTING DIABETES

Technical field

5 Embodiments of the invention are directed to compositions and methods for a short course of systemic treatment of diabetic patients with a gastrin/cholecystokinin (CCK) receptor ligand and an epidermal growth factor (EGF) receptor ligand, such treatment initiating pancreatic islet neogenesis when administered to subjects, and providing remission of diabetes for a prolonged period of time following cessation of treatment.

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Background

 About 800,000 people in the United States population suffer from insulin deficiency diabetes (also known as juvenile or type I diabetes), and about 30,000 new cases arise each year. Further, an extremely large and rapidly increasing number of patients have forms of
15 type II diabetes (also called adult onset or insulin-resistance diabetes), in this population at a level of epidemic proportions, that cause pancreatic exhaustion and insulin insufficiency. Diabetes type I is generally treated with insulin injection in response to blood glucose levels determined by patient glucose self-monitoring. A variety of forms of insulin, for example, slow and fast acting, and systems and devices suitable for insulin delivery by injection, for
20 example a delivery pen, are used by the growing population of diabetics.

 The abnormally high blood glucose (hyperglycemia) that characterizes diabetes, if left untreated, results in a variety of pathological conditions, for example, non-healing peripheral vascular ulcers, retinal damage leading to blindness, and kidney failure. Multiple daily doses of insulin administration by the patient, in response to frequent glucose self-monitoring per
25 day, are required on a lifetime basis, with concomitant expense and inconvenience to the patient and the health care system. Further, greater pathological consequences of diabetes are correlated with less rigorous control of blood glucose level, control being accomplished by glucose level monitoring and insulin administration, and by dietary restrictions, so that only the compliant patients avoid negative outcomes.

30 Alternative diabetes therapies to glucose monitoring and insulin injection have been sought, for example, in which the diabetes patient receives a transplant of pancreatic material. These possibilities offer the potential advantage of a normal physiological response, viz.,

rapid release of insulin in response to increased blood glucose concentration. Transplants of allogenic or xenograft pancreatic material, such as encapsulated porcine islets or islet duct cells that have been induced to differentiate in vitro, remain experimental. Transplantation of human pancreatic material remains limited by severe insufficiency of available material and immune rejection. Transplantation of allogenic or xenograft pancreatic islet tissue remains limited because of immune rejection of the transplant, or possible transmission of an animal virus.

Transgenic mice expressing both TGF α and gastrin recombinant genes have increased pancreatic islet mass, which does not occur in mice expressing either agent alone (U.S. patents 5,885,956, issued March 23, 1999 and 6,288,301, issued September 11, 2001). Therapeutic compositions and methods for controlling blood glucose and remediating insulin insufficiency, particularly compositions and methods that can cause rapid release of insulin in response to elevated blood glucose, and that are effective for an extended period of time, remain important medical priorities.

Summary

The invention is based in part on the discovery that administration to a diabetic subject of both a gastrin receptor ligand and an EGF receptor ligand, according to a short schedule of administration of the dosages, causes islet neogenesis in the subject, and produces a response that was a surprising long term remission of the diabetic condition. The period of remission is characterized by a lowering of blood glucose, and an increase in blood insulin. No cure involving in vivo islet regeneration in a diabetic patient is currently commercially available.

In one aspect, the invention herein provides a pharmaceutical composition including a first dose of a gastrin/cholecystokinin (CCK) receptor ligand, and a second dose of an epithelial growth factor (EGF) receptor ligand. The first and second doses are in a proportion in the composition that is effective for inducing islet neogenesis in a subject in need of islet neogenesis. The gastrin/CCK receptor ligand can be, for example, a synthetic gastrin derivative. The EGF receptor ligand can be a recombinant modified EGF. For example, in one embodiment, the gastrin/CCK receptor ligand is a synthetic gastrin derivative having a leucine substituted at position 15, and the EGF receptor ligand is a recombinant modified EGF having a deletion of two C-terminal amino acids and having a neutral amino acid substituted at position 51. The subject in need of islet neogenesis can be a patient with

diabetes. The diabetes can be, for example, insulin-dependent diabetes, for example, adult-onset diabetes.

In general, the first dose can be at least about equivalent by weight to the second dose in the composition. For example, the first dose is between about 2-fold and about 100-fold greater by weight than the second dose. In one embodiment, the first dose is between about 2-fold and about 10-fold by weight greater than the second dose. Alternatively, the first dose is between about 10-fold and about 100-fold by weight greater than the second dose. Specifically, the first dose can be about 0.1 microgram to about 1.0 microgram per kg body weight of the subject per day. Alternatively, the first dose can be about 1.0 microgram to about 10 micrograms per kg body weight of the subject per day; the first dose can be about 10 micrograms to about 100 micrograms per kg body weight of the subject per day; or the first dose can be about 100 micrograms to about 3.0 milligrams per kg body weight of the subject per day. In some embodiments, the dose per day (or 24 hour period) is divided among a plurality of administrations per day.

In general, the subject is a mammal. For example, the mammal is a rodent or a primate, for example, the subject is a human.

In one embodiment, the invention provides a pharmaceutical composition including a synthetic gastrin derivative and a recombinant modified EGF. The ratio of the gastrin derivative to the recombinant modified EGF is about 60:1. Yet another embodiment is a pharmaceutical composition having a synthetic gastrin derivative having a leucine substituted at position 15, and a recombinant modified EGF having a deletion of two C-terminal amino acids and a neutral amino acid substituted at position 51, wherein the ratio of the gastrin derivative to the modified EGF is about 60:1. Yet another embodiment can be a pharmaceutical composition for islet neogenesis therapy (I.N.T.TM), having an effective dose of at least about 1 µg/kg body weight of a modified recombinant EGF and at least about 30 µg/kg body weight of a synthetic gastrin derivative. Yet another embodiment is a pharmaceutical composition for I.N.T.TM, having an effective dose of at least about 1 µg/kg body weight of a recombinant modified EGF having a deletion of two C-terminal amino acids and a neutral amino acid substituted at position 51, and at least about 30 µg/kg body weight of a synthetic gastrin derivative having a leucine substituted at position 15.

In another aspect, an embodiment of the invention provides a method for treating a subject having diabetes, including: administering for a duration of treatment a composition having a first effective dose of a gastrin/CCK receptor ligand and a second effective dose of

an EGF receptor ligand, the first and second effective doses being suitable for inducing islet neogenesis in a subject in need of islet neogenesis, and causing a period of remission which is at least about four-fold greater than the duration of treatment. The composition can be effective in increasing blood insulin and reducing blood glucose for the period of remission.

5 For example, if the subject is a human, the duration of treatment is less than about 0.1 percent of the average human life span which is about 70-75 years, that is, the duration of treatment is less than about one month of treatment. Alternatively, the duration of treatment is less than about 0.2 percent of the average human life span; or the duration of treatment is less than about 0.3 percent of the average life span. Further, the
10 period of remission is at least about 0.5 percent of the average human life span; the period of remission is at least about one percent of the average human life span; or the period of remission is at least about two percent of the average human life span. The method in various embodiments includes repeating administering the composition at a time corresponding to about the end of the period of remission.

15 Yet another embodiment of the invention provides a method for treating a subject having insulin-dependent diabetes, including: administering a dose of each of a synthetic gastrin derivative and a recombinant modified EGF for a term of treatment which is shorter in duration than about one percent of an average lifespan of the subject species, the treatment resulting in increased insulin production and decreased blood glucose; and repeating
20 administering the composition at a time corresponding to about the end of the remission, thereby treating the subject having insulin-insufficient diabetes.

Yet another aspect of the invention provides a method for treating a subject having insulin-insufficient diabetes, including: administering a dose of each of a synthetic gastrin derivative having a leucine substituted at position 15, and a recombinant modified EGF
25 having a deletion of two C-terminus amino acids and having a neutral amino acid substituted at position 51, for a term of treatment which is shorter in duration than about one percent of an average lifespan of the subject species, the treatment resulting in increased insulin production and decreased blood glucose; and repeating administering the composition at a time corresponding to about the end of the remission, thereby treating the subject having
30 insulin-insufficient diabetes.

Yet another aspect of the invention provides a method of treating a diabetes patient in need of islet neogenesis, including: administering to the patient a composition having a first effective dose of a gastrin/CCK receptor ligand and a second effective dose of an EGF

receptor ligand, the composition being administered according to a dosing schedule of short duration; monitoring the blood glucose level in the patient following administering the composition; and reiterating administering of the composition to the patient when an increase in blood glucose level in the sample indicates that the patient is in need of further islet

5 neogenesis, such that the diabetes patient in need of islet neogenesis is treated. Accordingly, administering the composition causes increased blood insulin and decreased blood glucose. The duration of the dosing schedule is less than about three months; for example, the duration of the dosing schedule is less than about two months; for example, the duration of the dosing schedule is less than about one month. Further, monitoring the blood glucose level is less

10 frequent than about once daily; for example, monitoring the blood glucose level is less frequent than about once weekly. Monitoring the blood glucose level can be self-monitoring by the patient. Reiterating administering the composition can be less frequent than about once per six months. Alternatively, reiterating administering the composition can be less frequent than about once per year; or reiterating administering the composition can be less

15 frequent than about once per two years. In a related embodiment, the first effective dose of a gastrin/CCK receptor ligand in the composition is at least about ten-fold greater by weight than the second effective dose of the EGF receptor ligand.

In yet another aspect, the invention provides a method of treating a diabetes patient in need of islet neogenesis, including: administering to the patient a composition having a

20 synthetic gastrin derivative and a modified recombinant EGF, such that islet neogenesis is initiated and blood glucose is substantially reduced, the composition being administered according to a dosing schedule of less than about two months duration; monitoring the blood glucose level at intervals of less than about once per day; and reiterating administering the composition to the patient less frequently than about once per six months.

25 In yet another aspect, the invention provides a method of treating a diabetes patient in need of islet neogenesis, including: administering to the patient a composition including a synthetic gastrin derivative, the gastrin derivative having a leucine at position 15, and a recombinant modified EGF, the modified EGF having a deletion of two amino acids at the C-terminus and a neutral amino acid at position 51, such that islet neogenesis is initiated and

30 blood glucose is substantially reduced, the composition being administered according to a dosing schedule of less than about two months duration; monitoring the blood glucose level in a blood sample taken at intervals of less than about once per day; and reiterating administering the composition to the patient less frequently than about once per six months.

In yet another aspect, the invention provides a method of reducing insulin usage in an insulin-deficient diabetic patient, the method including: administering an effective dosage of an I.N.T.TM composition, to induce islet neogenesis; and reducing delivery of insulin as islet neogenesis is induced, thereby causing increased insulin secretion and decreased blood glucose. In a related embodiment, the composition comprises a gastrin/CCK receptor ligand and an EGF receptor ligand. The composition can have a gastrin/CCK receptor ligand in an amount that is at least about 10-fold greater in weight than an amount of an EGF receptor ligand in the composition. Administering the composition is performed according to a schedule less than about six months in duration. Further, reducing insulin delivery can be initiated after cessation of administering the composition. Alternatively, reducing exogenous insulin dosing can be initiated within the duration of the schedule of administering the composition. Thus, insulin delivery after administering the composition is reduced to less than about 70% compared to usage in the diabetic patient before administering the composition. For example, insulin delivery after administering the composition is reduced to less than about 50% compared to usage in the diabetic patient before administering the composition; or insulin delivery after administering the composition is reduced to less than about 10% compared to usage in the diabetic patient prior to administering the composition; or insulin delivery after administering the composition is reduced to less than about 1% compared to usage in the diabetic patient prior to administering the composition.

Yet another aspect of the invention provided is a method of reducing insulin usage in an insulin-dependent diabetic patient, the method including: administering an effective dosage of a gastrin/CCK receptor ligand and an effective dosage of an EGF receptor ligand in a proportion of at least about 10:1; and reducing insulin delivering following onset of reduction of blood glucose in the patient.

An embodiment of the invention herein provides a method of increasing islet neogenesis in a patient, including administering over a term of short duration a gastrin/CCK receptor ligand and an EGF receptor ligand, such that islet neogenesis is increased.

An embodiment of the invention herein provides a kit including a gastrin/CCK receptor ligand and an EGF receptor ligand. For example, the gastrin/CCK receptor ligand and the EGF receptor ligand are combined in a single container. The kit can contain an instruction manual or a set of instructions. Further, the gastrin/CCK receptor ligand and the EGF receptor ligand can be present as unit dosages. The kit can further include insulin.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief description of the drawings

5 Figure 1 is a graph showing improved glucose tolerance of STZ diabetic rats after treatment by islet neogenesis therapy (I.N.T.TM) with gastrin and TGF α , as a function of time. Rat groups are non-diabetic controls (labeled No STZ control n = 4, in the Figure); diabetic STZ mice treated with vehicle only (labeled STZ Vehicle n=12); rats treated with a low I.N.T.TM dosage of 4 μ g of each of gastrin and TGF α per kg body weight (labeled STZ 4
10 TGF/gastrin n=6); and rats treated with 40 μ g of each of gastrin and TGF α per kg body weight, a 10-fold higher I.N.T.TM dosage of each of gastrin and TGF α (labeled STZ 40 TGF/gastrin n=8). The data show that both groups of the I.N.T.TM treated STZ rats achieved a lower blood glucose level following a challenge than diabetic rats in the untreated control group.

15 Figure 2 is a bar graph showing increased insulin release in I.N.T.TM treated rats 15 and 30 min following a glucose challenge. The left set of three bars each drawn as empty bars, stippled bars, and solid bars show plasma insulin levels (ng/ml) in each of the three STZ diabetic rats as in Figure 1. The insulin levels in the non-diabetic rat group at 15 and 30 min following challenge is shown in the bars on the right, on a different scale. The data show that
20 diabetic rats administered the I.N.T.TM treatment at the high I.N.T.TM dosage of Figure 1 produce more than three-fold greater insulin than untreated diabetic rats at 15 and 30 min after the challenge, and that rats receiving the low I.N.T.TM dosage of Figure 1 make more than four-fold more insulin than untreated diabetic rats at 30 min.

 Figure 3 is a set of graphs showing blood glucose levels for the groups of rats as in
25 Figure 1, assayed as a function of time, in blood samples taken two weeks after treatment (left panel) and eight weeks after treatment (right panel). The data show that blood glucose in the low dose I.N.T.TM treated rats remains lower than in untreated controls following a glucose challenge for at least eight weeks following treatment.

 Figure 4 is a bar graph showing fasting blood glucose concentration, in mM, in non-
30 diabetic rats (labeled Control), diabetic untreated rats (labeled Vehicle), and rats administered low dosage I.N.T.TM treatment (labeled TGF/GAS4), at two, four and eight weeks following treatment. Data show that the I.N.T.TM-treated rats remain capable of maintaining normal fasting blood glucose levels for at least eight weeks following treatment.

Figure 5 is a graph showing sustained improvement in growth of diabetic I.N.T.TM-treated rats for at least eight weeks following treatment, compared to untreated diabetic control rats.

Figure 6 is a graph showing fasting blood glucose levels of diabetic rats (labeled Diabetic Control), non-diabetic untreated rats (labeled Non-diabetic Control), and rats administered low dosage I.N.T.TM treatment (labeled TGF/GAS4), as function of time after treatment (2, 4, 8 and 16 weeks). Data show that I.N.T.TM-treated rats maintain a lower fasting blood glucose level at least for 16 weeks following treatment, compared to untreated diabetic control rats

Figure 7A is a bar graph showing fasting plasma insulin C peptide concentrations and fasting blood glucose concentrations. Figure 7B is a bar graph showing pancreatic insulin content and β cell mass determined morphometrically. Insulin dependent STZ diabetic rats (BW > 300 g) were treated by subcutaneous continuous infusion for two weeks with a synthetic human gastrin derivative, gastrin 17 (Leu 15), 270 μ g/kg per day, and with recombinant human EGF 1-51gln51, 227 μ g/kg per day, or with a Vehicle control. Each bar show mean \pm SE, for each group of 6 animals. * denotes $p < 0.05$ (Student's t test, unpaired).

Figure 8 is scattergram graph showing levels of fasting blood glucose concentrations from each of the STZ insulin dependent diabetic rats at 4, 11 and 14 days after completing treatment with gastrin, 300 μ g/kg, and EGF, 100 μ g/kg (group A); with 3,000 μ g/kg gastrin and 100 μ g/kg EGF (Group C); or control Vehicle (Group V), injected subcutaneously three times daily for 28 days. The number of animals showing remission of diabetes, remission indicated by having a blood glucose level of less than 10 mM at 14 days post treatment, was compared between groups by Chi squared test at a significance of $p < 0.05$. The graph shows that at 14 days after 28 days treatment with 3000 μ g/kg and EGF 100 μ g/kg (group C), a significant number of animals (4 of 10) showed complete remission of diabetes compared both to the vehicle group (where none of 9 animals showed remission) and to the experimental group having a different ratio of 300 μ g/kg and EGF 100 μ g/kg (group A, where only one animal showed complete remission). A feature of this response is that remission was delayed 11-14 days after treatment was completed.

Detailed description of the embodiments

As used herein, the term "gastrin/CCK receptor ligand" encompasses compounds that stimulate the gastrin/CCK receptor such that when EGF receptors in the same or adjacent tissue or in the same individual are also stimulated, neogenesis of insulin-producing pancreatic islet cells is induced. Examples of such gastrin/CCK receptor ligands are given in U.S. patent 6,288,301 issued Sept. 11, 2001, and include various forms of gastrin, such as gastrin 34 (big gastrin), gastrin 17 (little gastrin), and gastrin 8 (mini gastrin); various forms of cholecystokinin such as CCK 58, CCK 33, CCK 22, CCK 12 and CCK 8; and other gastrin/CCK receptor ligands that demonstrate the same synergistic activity with EGF receptor ligands, and which can induce differentiation of cells of mature pancreas to form insulin-secreting islet cells, when acting synergistically with an EGF receptor ligand.

Small forms of gastrin such as gastrin 17 are economically prepared by peptide synthesis, and the synthetic peptides are commercially available. Synthetic human gastrin 17, and derivatives such as human gastrin 17 having methionine substituted for leucine at position 15 are also available from Bachem AG, Bubendorf, Switzerland, and from Researchplus.

Gastrin/CCK receptor ligands include also active analogs, fragments and other modifications of the above ligands. Such ligands also include compounds that increase the secretion of endogenous gastrins, cholecystokinins or similarly active peptides from sites of tissue storage. Examples of these are omeprazole which inhibits gastric acid secretion, and soya bean trypsin inhibitor which increases CCK stimulation.

As used herein, the term "EGF receptor ligand" encompasses compounds that stimulate the EGF receptor such that when gastrin/CCK receptors in the same or adjacent tissue or in the same individual are also stimulated, neogenesis of insulin-producing pancreatic islet cells is induced. Examples of such EGF receptor ligands include full length EGF which is EGF1-53, and further include EGF1-48, EGF1-49, EGF1-52, and fragments and active analogs thereof. Other examples of EGF receptor ligands are TGF α forms that include 1-48, 1-47, 1-51, and amphiregulin and pox virus growth factor as well as any EGF receptor ligands that demonstrate the same synergistic activity with gastrin/CCK receptor ligands. These include active analogs, fragments and modifications of the above. For further background, see, Carpenter and Wahl, Chapter 4 in Peptide Growth Factors (Eds. Sporn and Roberts), Springer Verlag, 1990.

- EGF has mitogenic activity, i.e., it stimulates the growth of various cells and tissues (Karnes *supra*; Carpenter, G. et al. *J. Cell Physiol* 88, 227-237, 1976; Gasslander, T. et al. *Eur. Surg. Res.* 29, 142-149, 1997). EGF is also found to have a cytoprotective effect, stimulating migration of a cell toward a wound *in vivo*, or toward a gap introduced in a monolayer of cells in culture, to promote wound healing. These biological activities are specific to a family of structurally related growth factors, including EGF, TGF α , amphiregulin and heparin binding EGF-like growth factor (Karnes, *supra*). The members of this family of growth factors have identical amino acids at 11 residues of the amino acid sequence, six of which are cysteine residues that form disulfide bonds.
- The group of compounds which are EGF receptor ligands further includes "modified EGF", which are variants of normal or wild type EGF. Modifications have been shown to affect one or more biological activity such as the rate of clearance of EGF. The term includes peptides having the an amino acid sequence substantially similar to that of human EGF, for example, with one or a few amino acid substitutions at various residue positions.
- Recombinant EGF forms have been genetically engineered to have alterations in structure and activities, for example, EGF having a methionine at position 21 replaced by a leucine residue has been described (U.S. patent number 4,760,023). Recombinant human EGF (hEGF) having 51 residues, i.e., lacking the two C-terminal residues at positions 52 and 53 of hEGF, and having a neutral amino acid substitution at position 51, retain EGF activity and are more resistant to protease degradation during a microbial production process, and following administration to a subject. A series of nucleic acid molecules have been described that encode a family of proteins that have significant similarity to EGF and TGF α (WO 00/29438). EGF muteins (mutated EGF) having histidine at residue 16 replaced with a neutral or acidic amino acid have been described (WO 93/03757), such forms retaining activity at low values of pH. Chemical analogues and fragments of EGF and TGF α retain ability to bind various members of the EGF receptor family (U.S. patent number 4,686,283). Various modifications of EGF or TGF α confer advantageous properties affecting one or more of recombinant protein production, in vitro and in vivo stability, and in vivo activity. A preferred recombinant modified EGF receptor ligand of the embodiments herein retains substantially full I.N.T.TM activity, and has in vivo and/or in vitro stability that is that is at least about as great or greater than normal or wild type hEGF.

A principal aspect of the invention is a method for treating diabetes mellitus in an individual in need thereof by administering to the individual a composition including a

gastrin/CCK receptor ligand and an EGF receptor ligand, each in a sufficient amount and in proportion to each other so that the combination of these ligands effects differentiation of pancreatic islet precursor cells to mature insulin-secreting cells, a process known as islet neogenesis. The cells that differentiate are latent islet precursor cells that are present in the pancreatic duct of the mature animal subject. See U.S. patent number 5,885,956, issued March 23, 1999.

The term "mature" as used herein refers exclusively to a post-natal animal, to distinguish the mature animal from a fetal animal. Mature refers to a mammal of chronological age older than 5% of the average lifespan of the species of mammal. This term is unrelated to a stage of the subject's development or to a time of onset of diabetes. In one embodiment, the methods and compositions herein are useful for treating a patient having type I diabetes mellitus, i.e., a form of diabetes characterized by insulin dependency including insulin absence or deficiency, and referred to as "juvenile" diabetes. The method and compositions herein are useful also for treatment of patients with insulin-dependent type II, or adult-onset diabetes, for example, who produce insufficient insulin to maintain blood glucose levels due to pancreatic exhaustion.

One embodiment of a method herein comprises administering, preferably systemically, a composition which is differentiation regenerative amount of a gastrin/CCK receptor ligand, for example, gastrin, and an EGF receptor ligand, for example, EGF or TGF α , to the individual. The gastrin is synthetic, for example, synthetic human gastrin, for example, human gastrin 17, for example, having leucine at residue 15. The EGF receptor ligand is hEGF, for example, recombinantly produced EGF, for example, a recombinant modified EGF, for example, EGF produced recombinantly from a synthetic gene carrying modifications, such as a deletion of two C-terminal amino acids at positions 52 and 53, and encoding an EGF having a length of 51 amino acids. Further EGF modification can include one or more of a variety of substitutions, such as a substitution of a neutral amino acid at position 51 rather than the normal residue at position 51 of hEGF, which is the charged amino acid glutamic acid. A neutral amino acid as defined herein is one that is uncharged at physiological pH, and is preferably hydrophilic, such as by substitution of serine, alanine, and glutamine for the residue normally found at that position.

In another embodiment, the invention relates to a method for effecting the differentiation of pancreatic islet precursor cells of a subject, for example, a mammal, by stimulating such cells with a combination of a gastrin/CCK receptor ligand, for example, a

gastrin such as gastrin 17, for example, synthetic gastrin 17 carrying leucine at position 15, and an EGF receptor ligand, for example, a TFG α , or a recombinant modified EGF, according to a particular dosing schedule.

As used herein, a dosing schedule refers to a protocol for administering an I.N.T.TM composition, and includes the amount of the composition delivered per day, and the duration or period of time over which the composition is administered.

Most insulin dependent diabetic patients require insulin injection at least on a daily basis. Multiple doses per day of insulin are required under certain circumstances of illness or diet for management of diabetes, and the insulin administration is indicated by results of frequent glucose monitoring, another activity which is required of a diabetes patient for optimal management of the disease, which is performed for example as often as five times daily.

In contrast, the compositions and methods herein are used for a dosage schedule of short duration, for example, a dosage period of less than 0.5 percent, or 0.2 percent, or 0.1 percent of a subject species' average lifespan.

Administration over a short duration dosing schedule of the compositions herein is followed by an extended period of efficacy, even complete remission of disease symptoms. Lifespan data used herein include: an average lifespan of a rodent in captivity of about 2.5 years (30 months); an average lifespan of a primate that is a macaque such as a cynomolgus monkey (*Macaca fascicularis*) in captivity of about 30 years (360 months); an average lifespan of a primate that is a chimpanzee (*Pan troglodytes*) in captivity of about 45 years (540 months); and an average lifespan of a primate that is a human of about 70-75 years (840-900 months). It is recognized that an average lifespan of a species varies with conditions that are cultural, such as diet, and/or genetic.

The short term of duration of the dosage schedule for a rodent such as a rat is calculated herein as, for example, equal or less than about 28 days, about 18 days, or less than about 9 days. The duration of a dosage schedule for a primate, for example, a cynomolgus monkey, a chimpanzee or a human, is longer than for a rodent, e.g., the treatment period is less than about one year, less than about three months, less than about two months, or even less than about one month.

The short term of duration of the dosage schedule for a human patient represents and even smaller percentage of the lifespan. For example, a four week duration of treatment is 0.11% of a lifespan of 70 years (3640 weeks); a two week treatment is 0.05% of the lifespan.

Efficacy of the treatment extends to a period of 26 weeks (0.7%), or 52 Weeks (1.4%), or 104 weeks (2.86%).

As a result of administration of the I.N.T.TM compositions provided herein according to a dosage schedule of such short duration, the process of islet neogenesis is initiated.

5 Precursor cells in the subject are induced to differentiate, and the differentiating cells then mature into islet cells capable of secreting insulin in response to fluctuations in blood glucose levels, i.e., a subject with diabetes enters a period of remission characterized by a normal response to a blood glucose challenge. As a result of this administration, remission of diabetes is initiated, so that the standard dosage of insulin given to a diabetic patient prior to
10 therapy is reduced, as determined by the level of blood glucose obtained by monitoring, for example, by self-monitoring by the patient, during and following treatment. Remission from diabetes due to successful islet neogenesis therapy is indicated by a decreased fasting blood level of glucose, and by a decreased level and duration of elevated blood glucose in response to a dietary challenge of sugar consumption. Upon achieving successful islet neogenesis,
15 insulin administration is reduced from, for example, five injections to two injections per day; from two injections to one injection per day; and from one to none, as indicated by data obtained from monitoring blood glucose levels. One of ordinary skill in the art of pharmacology, when treating a diabetic patient, is familiar with adjusting insulin dosage to levels of blood glucose following fasting and under other physiological conditions.

20 Dosages of the I.N.T.TM compositions to be administered to a subject are adjusted for known variations from species to species in standard data encompassing criteria for absorption, distribution, half-life kinetics in circulation, metabolism, excretion, and toxicology of the receptor ligands of the embodiments herein, for example, for each primate and rodent species. In general, dosages are adjusted to be about 100-fold greater for
25 administration to a rodent species than to a primate species. For example, a dose of an I.N.T.TM composition for a rat is exemplified by about 3,000 µg/day of a gastrin/CCK receptor ligand and about 100 µg of an EGF receptor ligand, administered for example in three injections per day (for a total of about 9,000 µg of gastrin/CCK receptor ligand and about 300 µg of EGF receptor ligand per day), on a per kg of body weight basis. For a
30 primate such as a cynomolgus monkey, a chimpanzee, or a human, the comparable dose is, for example, about 1 to about 3 µg of EGF receptor ligand, or about 3 to about 10 µg of EGF receptor ligand per kg body weight, and about 30 to about 90 µg, or about 90 to about 300 µg of gastrin/CCK receptor ligand per kg body weight, such daily doses to be administered as a

total bolus given once per day, or divided into subdoses to be administered in two or administrations per day.

Modes of administration of the receptor ligands include but are not limited to subcutaneous, transdermal, intramuscular, intraperitoneal, intravenous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by pump, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.). The receptor ligands herein may be administered in combination with one or a plurality of other biologically active agents. Administration is preferably systemic.

10 The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable sterile carrier or excipient. Such a carrier includes but is not limit to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

15 The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, such as Tween, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, 20 magnesium carbonate, etc.

Various delivery systems are known and can be used to administer a therapeutic of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules and the like.

In a preferred embodiment, the composition is formulated in accordance with routine 25 procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Compositions for intravenous administration are typically solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include additional components, for example, a solubilizing agent. Generally, the ingredients are supplied either separately or pre-mixed in unit dosage form. In addition to solution form, the composition can be supplied as a dry lyophilized powder, or as a non-aqueous concentrate, for example, in a hermetically 30 sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed using an infusion bottle containing sterile pharmaceutical grade water or saline. Where the

composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

The therapeutic agent embodiments of compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of each of the therapeutic agents of the compositions herein, and their relative amount with respect to each other which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. The precise dose to be employed in a formulation depends also on route of administration, and the extent of the disease or disorder in a given patient, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous and subcutaneous administration are generally about 0.1 microgram or one microgram, to about one milligram or three milligrams, per kilogram body weight, for example, from about 20 to about 500 micrograms of each active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg per kg body weight, to about 1 mg per kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. A daily dose is administered as a single dose or divided into a plurality of smaller fractional doses, to be administered several times during the day.

Suppositories generally contain active ingredient in the range of about 0.5% to about 10% by weight; oral formulations preferably contain about 10% to about 95% active ingredient by weight.

Embodiments of the invention herein also provide a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention, for example, a container having a unit dosage of each or both of a gastrin/CCK receptor ligand and an EGF receptor ligand. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The pack or kit can in certain embodiments include one or more containers having insulin, to be administered during the dosing schedule prior to induction of islet neogenesis and remission from diabetes.

5

EXAMPLES

Example 1: Prolonged efficacy of I.N.T.TM compositions and methods in diabetic rats

Diabetes was induced by intravenous injection with 50mg/kg streptozotocin (STZ), which reduced pancreatic insulin content by 95%. One week later, STZ diabetic rats (of weight 200-250 g) were administered either the vehicle alone as a control (labeled CON in Figure 1), or were administered a combination of human gastrin 17 (4 µg/kg) and human TGFα (4µg/kg) peptides (labeled GAS/TGF) by once-daily, intraperitoneal injections for 12 days. At 1, 2, 4 and 8 weeks after treatment, intraperitoneal glucose tolerance tests (IPGTT) were performed, to measure both blood glucose levels, and plasma insulin levels, in response to a challenge amount of glucose.

The data show that GAS/TGF treatment stimulated β islet cell neogenesis, as demonstrated morphologically by a significant increase in the number of small clusters of insulin staining cells adjacent to pancreatic ducts. Further, GAS/TGF treated rats had significantly increased pancreatic insulin concentrations compared to CON rats. At all times studied, IPGTT showed that the GAS/TGF treated rats had significantly lower blood glucose levels than the CON rats (see Figure 1). Further, the glucose-stimulated increase in plasma insulin levels was 2-3 fold greater in the GAS/TGF treated rats compared to CON rats (Figure 2).

Surprisingly, prolonged efficacy of the treatment was observed, with improved glucose tolerance in response to a glucose challenge in the GAS/TGF rats maintained at least to 8 weeks after cessation of treatment (Figure 3). Further, 8 weeks after cessation of treatment, the GAS/TGF treated rats also displayed normal fasting blood glucose levels (6.4 ± 1.2 mM) compared to the diabetic levels (12.0 ± 2.2 mM) observed in the CON rats (see Figure 4). Rat growth in the I.N.T.TM-treated groups was normal (Figure 5). The improved glucose tolerance correlated with improved body weight gain by the GAS/TGF group during the 8 week post treatment period.

Further, additional data indicate that efficacy of the I.N.T.TM treatment continues to at least 16 weeks after cessation of the treatment (Figure 6).

Example 2: Treatment by subcutaneous administration of EGF and gastrin in diabetic rats for two weeks results in increased pancreatic β cell mass, insulin content, and insulin secretion, and reduced blood glucose in diabetic rats.

Diabetic STZ rats were prepared as above, using rats of body weight greater than 300g. These were treated with EGF and gastrin by subcutaneous administration (subcutaneous continuous infusion), as follows. Rats were treated for two weeks with synthetic human gastrin 17 (Leu 15), 270 $\mu\text{g/kg}$ per day and recombinant human EGF 1-51 gln 51, 227 $\mu\text{g/kg}$ per day, or with Vehicle control. Each bar show mean \pm SE for 6 animals, and * denotes $p < 0.05$ (Student's t test, unpaired). Pancreatic insulin was determined by immunoassay, and β cell mass was determined by point counting morphometric analysis of pancreatic histological sections after immunohistochemical staining with insulin antibodies to stain β cells. Antibody is specific for insulin C peptide.

Increased plasma insulin C peptide levels (Fig 7A, left panel) and reduced fasting blood glucose levels were observed (Fig 7A, right panel) were observed for the EGF/gastrin treated rats, compared to control rats receiving vehicle only.

Further, rats were examined for pancreatic insulin content and number of insulin secreting pancreatic β cells (see Fig. 7, Panel B). Figure 7B shows the pancreatic insulin content and β cell mass determined morphometrically, from the treated insulin dependent STZ diabetic rats (labeled INT) and from vehicle control (labeled Veh). Treated rats showed about three fold greater insulin content, and about two fold greater β cell mass than control rats.

Example 3: Increasing the proportion of gastrin in the EGF/gastrin combination improved the remission of diabetes.

I.N.T.TM compositions and methods of treatment were performed using groups of STZ rats, using a dosing schedule of 28 days duration, and a composition having gastrin at a weight 30-fold greater than EGF. Rats were administered 100 micrograms/injection of the EGF receptor ligand per kg body weight, and 3 milligrams/injection of gastrin/CCK receptor ligand. Three such injections were administered per day.

Fig. 8 shows the fasting blood glucose level after 28 days of treatment of diabetic rats with 3,000 $\mu\text{g/kg}$ synthetic gastrin 17 Leu15, administered along with recombinant modified EGF51 Gln51 (100 $\mu\text{g/kg}$). The data show that this treatment gave a significantly greater number of rats with remission of diabetes (as defined by fasting blood glucose below 10 mM)

(Group C) compared to the untreated control group (Group V). By contrast, treatment with a 300 µg/kg gastrin with the same dose of EGF (100 µg/kg) did not induce remission in a significant number of animals (Group A).

- Surprisingly, within 14 days of cessation of treatment with the I.N.T. composition
- 5 having a ratio by weight of gastrin/EGF of 30:1, blood glucose levels of a significant number of treated animals were observed to fall within non-diabetic levels. The 30:1 level of gastrin was important to the response since the control group of diabetic rats treated with 3:1 ratio having a 10-fold lower dose of gastrin did not show a significant number of non-diabetic animals at 14 days after treatment.

10

What is claimed is:

1. A pharmaceutical composition comprising a first dose of a gastrin/cholecystokinin (CCK) receptor ligand, and a second dose of an epithelial growth factor (EGF) receptor ligand, wherein the first and second doses are in a proportion in the composition that is effective for inducing islet neogenesis in a subject in need of islet neogenesis.
2. The composition of claim 1, wherein the gastrin/CCK receptor ligand is a synthetic gastrin derivative.
3. The composition of claim 1, wherein the EGF receptor ligand is a recombinant modified EGF.
4. The composition of claim 1, wherein the gastrin/CCK receptor ligand is a synthetic gastrin derivative having a leucine substituted at position 15, and the EGF receptor ligand is a recombinant modified EGF having a deletion of two C-terminal amino acids and having a neutral amino acid substituted at position 51.
5. The composition of claim 1, wherein the subject in need of islet neogenesis is a patient with diabetes.
6. The composition of claim 5, wherein the diabetes is insulin-dependent diabetes.
7. The composition of claim 5, wherein the diabetes is adult-onset diabetes.
8. The composition of claim 1, wherein an amount of the first dose is at least about equivalent by weight to an amount of the second dose in the composition.
9. The composition of claim 1, wherein the first dose is between about 2-fold and about 100-fold greater by weight than the second dose.
10. The composition of claim 1, wherein the first dose is between about 2-fold and about 10-fold by weight greater than the second dose.
11. The composition of claim 1, wherein the first dose is between about 10-fold and about 100-fold by weight greater than the second dose.

12. The composition of claim 1, wherein the first dose is about 0.1 microgram to about 1.0 microgram per kg body weight of the subject per day.
13. The composition of claim 11, wherein the first dose is about 1.0 microgram to about 10 micrograms per kg body weight of the subject per day.
- 5 14. The composition of claim 11, wherein the first dose is about 10 micrograms to about 100 micrograms per kg body weight of the subject per day.
15. The composition of claim 11, wherein the first dose is about 100 micrograms to about 10 milligrams per kg body weight of the subject per day.
16. The composition of claim 1, wherein the subject is a mammal.
- 10 17. The composition of claim 16, wherein the mammal is a rodent or a primate.
18. The composition of claim 1, wherein the subject is a human.
19. The composition of any of claims 12-15, wherein the dose per day is divided into a plurality of administrations per day.
20. A pharmaceutical composition comprising a synthetic gastrin derivative and a
15 recombinant modified EGF, wherein the ratio of the gastrin derivative to the recombinant modified EGF is about 60:1.
21. A pharmaceutical composition comprising a synthetic gastrin derivative having a leucine substituted at position 15, and a recombinant modified EGF having a deletion of two C-terminal amino acids and a neutral amino acid substituted at position 51,
20 wherein the ratio of the gastrin derivative to the modified EGF is about 60:1.
22. A pharmaceutical composition for islet neogenesis therapy (I.N.T.TM), comprising an effective dose of at least about 1 µg/kg body weight of a modified recombinant EGF and at least about 30 µg/kg body weight of a synthetic gastrin derivative.
23. A pharmaceutical composition for I.N.T.TM, comprising an effective dose of at
25 least about 1 µg/kg body weight of a recombinant modified EGF having a deletion of two C-terminal amino acids and having a neutral amino acid substituted at position 51, and at least

about 30 µg/kg body weight of a synthetic gastrin derivative having a leucine substituted at position 15.

24. A method for treating a subject having diabetes, comprising: administering for a duration of treatment a composition having a first effective dose of a gastrin/CCK receptor ligand and a second effective dose of an EGF receptor ligand, the first and second effective doses being suitable for inducing islet neogenesis in a subject in need of islet neogenesis, and causing a period of remission which is at least about four-fold greater than the duration of treatment.

25. The method of claim 24, wherein the composition is effective in increasing blood insulin and reducing blood glucose for the period of remission.

26. The method of claim 24, wherein the composition of the first and second effective doses are mixed and administered as a single injection.

27. The method of claim 24, wherein the composition of the first and second effective doses are mixed and administered as a single subcutaneous injection.

28. The method of claim 24, wherein treatment reduces the dose of exogenous insulin needed to prevent hyperglycemia in an insulin-dependent diabetic.

29. The method of claim 24, wherein the subject is a human, and a duration of treatment is less than about 0.3 percent of an average human life span.

30. The method of claim 29, wherein the duration of treatment is less than about 0.2 percent of the average human life span.

31. The method of claim 30, wherein the duration of treatment is less than about 0.1 percent of the average human life span.

32. The method of claim 24, wherein the subject is a human, and a period of remission is at least about 0.5 percent of an average human life span.

33. The method of claim 24, wherein the period of remission is at least about one percent of an average human life span.

34. The method of claim 24, wherein the period of remission is at least about two percent of an average human life span.

35. The method of claim 24, further comprising repeating administering the composition at a time corresponding to about the end of the period of remission.

5 36. A method for treating a subject having insulin-insufficient diabetes, comprising:
 administering a dose of each of a synthetic gastrin derivative and a recombinant modified EGF, for a term of treatment which is shorter in duration than about one percent of an average lifespan of the subject species, the treatment resulting in a remission of the
10 diabetes wherein the subject has increased blood insulin and decreased blood glucose; and
 repeating administering the composition at a time corresponding to about the end of the remission, thereby treating the subject having insulin-insufficient diabetes.

 37. A method for treating a subject having insulin-insufficient diabetes,
15 comprising:
 administering for a term of treatment which is shorter in duration than about one percent of an average lifespan of the subject species a dose of each of a synthetic gastrin derivative having a leucine substituted at position 15, and a recombinant modified EGF having a deletion of two C-terminus amino acids and a having neutral amino acid substituted
20 at position 51, the treatment resulting in a remission of the diabetes wherein the subject has increased blood insulin and decreased blood glucose; and
 repeating administering the composition at a time corresponding to about the end of the remission, thereby treating the subject having insulin-insufficient diabetes.

25 38. A method of treating a diabetes patient in need of islet neogenesis, comprising:
 administering to the patient a composition comprising a first effective dose of a gastrin/CCK receptor ligand and an a second effective dose of an EGF receptor ligand, the composition being administered according to a dosing schedule of short duration;
30 monitoring the blood glucose level in the patient following administering the composition; and

reiterating administering the composition to the patient when an increase in blood glucose level indicates that the patient is in need of further islet neogenesis, such that the diabetes patient in need of islet neogenesis is treated.

5 39. The method of claim 38, wherein administering the composition causes increased blood insulin and decreased blood glucose.

 40. The method of claim 38, wherein the duration of the dosing schedule is less than about three months.

10 41. The method of claim 40, wherein the duration of the dosing schedule is less than about two months.

 42. The method of claim 41, wherein the duration of the dosing schedule is less than about one month.

 43. The method of claim 38, wherein monitoring the blood glucose level is less
15 frequent than about three times daily.

 44. The method of claim 43, wherein monitoring the blood glucose level is less frequent than about once daily.

 45. The method of claim 46, wherein monitoring the blood glucose level is less frequent than about once weekly.

20 46. The method of claim 35, wherein monitoring the blood glucose level is self-monitoring by the patient.

 47. The method of claim 38, wherein reiterating administering the composition is less frequent than about once per six months.

 48. The method of claim 38, wherein reiterating administering the composition is
25 less frequent than about once per year.

 49. The method of claim 38, wherein reiterating administering the composition is less frequent than about once per two years.

50. The method of claim 38, wherein the first effective dose of a gastrin/CCK receptor ligand in the composition is at least about ten-fold greater by weight than the second effective dose of the EGF receptor ligand.

5 51. A method of treating a diabetes patient in need of islet neogenesis, comprising:
administering to the patient a composition comprising a synthetic gastrin derivative and a modified recombinant EGF, such that islet neogenesis is initiated and blood glucose is substantially reduced, the composition being administered according to a dosing schedule of less than about two months duration;
10 monitoring the blood glucose level at intervals of less than about once per day; and reiterating administering the composition to the patient less frequently than about once per six months.

15 52. A method of treating a diabetes patient in need of islet neogenesis, comprising:
administering to the patient a composition comprising a synthetic gastrin derivative having a leucine at position 15, and a recombinant modified EGF having a deletion of two C-terminus amino acids and having a neutral amino acid substituted at position 51, such that islet neogenesis is initiated and blood glucose is substantially reduced, the composition being
20 administered according to a dosing schedule of less than about two months duration;
monitoring the blood glucose level at intervals of less than about once per day; and reiterating administering the composition to the patient less frequently than about once per six months.

25 53. A method of reducing exogenous insulin usage in an insulin-dependent diabetic patient, the method comprising:
administering an effective dosage of an I.N.T.TM composition, to induce islet neogenesis; and
reducing the dose of exogenous insulin needed to prevent high blood glucose,
30 whereby blood glucose is decreased.

54. The method of claim 53, wherein the composition comprises a gastrin/CCK receptor ligand and an EGF receptor ligand.

55. The method of claim 54, wherein the composition comprises the gastrin/CCK receptor ligand in an amount that is at least about 10-fold greater in weight than an amount of the EGF receptor ligand.

56. The method of claim 53, wherein administering the composition is performed according to a schedule less than about three months in duration.

57. The method of claim 56, wherein reducing insulin delivery is initiated after cessation of administering the composition.

58. The method of claim 57, wherein reducing insulin delivery is initiated within the duration of the schedule of administering the composition.

59. The method of claims 57 or 58, wherein insulin delivery after administering the composition is reduced to less than about 70% compared to usage in the diabetic patient before administering the composition.

60. The method of claims 57 or 58, wherein insulin delivery after administering the composition is reduced to less than about 50% compared to usage in the diabetic patient before administering the composition.

61. The method of claims 57 or 58, wherein insulin delivery after administering the composition is reduced to less than about 10% compared to usage in the diabetic patient prior to administering the composition.

62. The method of claims 57 or 58, wherein insulin delivery after administering the composition is reduced to less than about 1% compared to usage in the diabetic patient prior to administering the composition.

63. A method of reducing insulin usage in an insulin-dependent diabetic patient, the method comprising:

administering an effective dosage of a gastrin/CCK receptor ligand and an effective dosage of an EGF receptor ligand in a proportion of at least about 10:1; and

reducing insulin delivering following onset of reduction of blood glucose in the patient.

64. A method of increasing islet neogenesis in a patient, comprising administering
5 over a term of short duration a gastrin/CCK receptor ligand and an EGF receptor ligand, such that islet neogenesis is increased.

65. A kit comprising a gastrin/CCK receptor ligand and an EGF receptor ligand.

66. The kit of claim 65, wherein the gastrin/CCK receptor ligand and the EGF
10 receptor ligand are combined in a single container.

67. The kit of claim 65 or 66, wherein the gastrin/CCK receptor ligand and the EGF receptor ligand are present as unit dosages.

68. The kit of claim 65, further comprising insulin.

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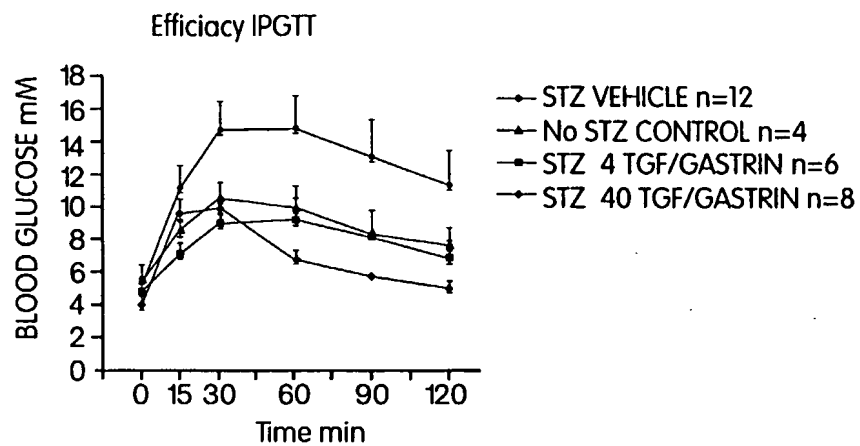


Fig. 1

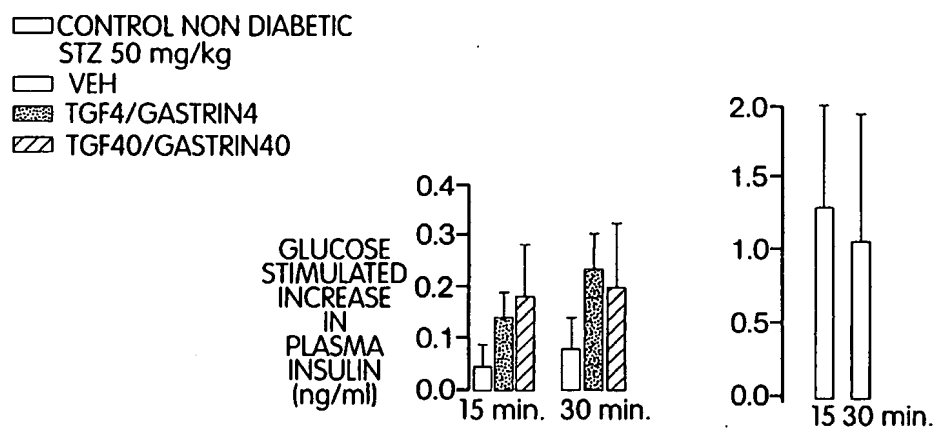


Fig. 2

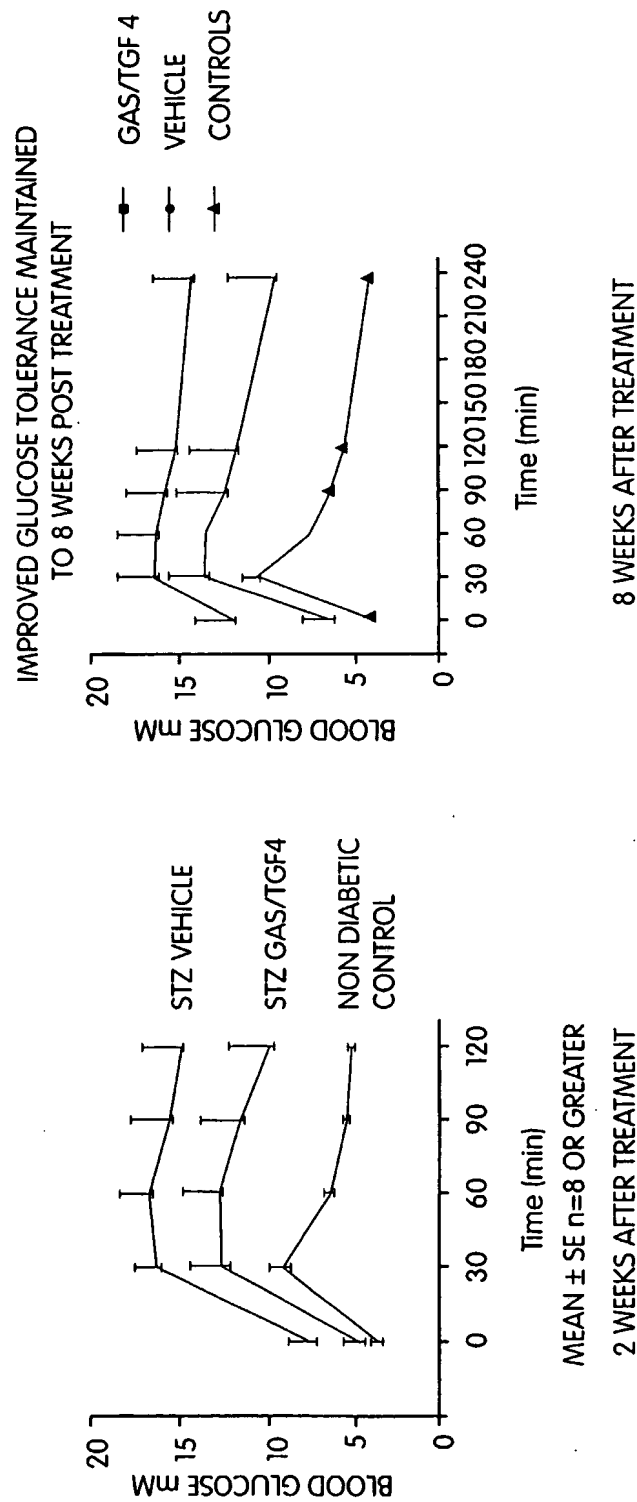


Fig. 3

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Fig. 4

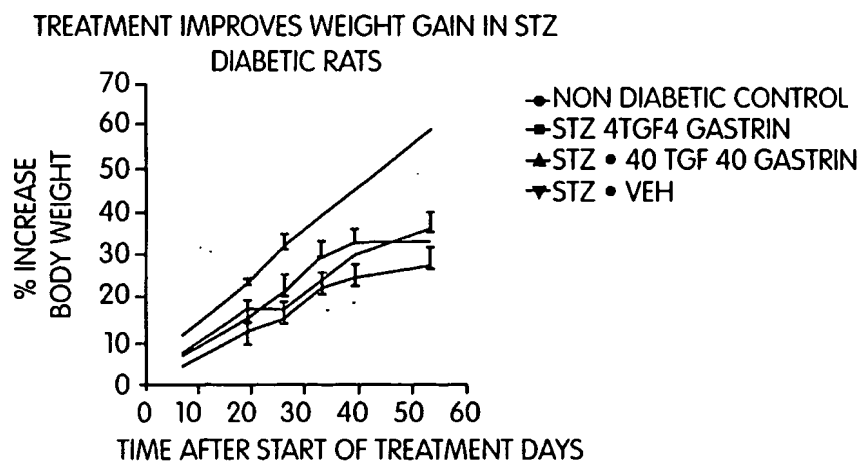


Fig. 5

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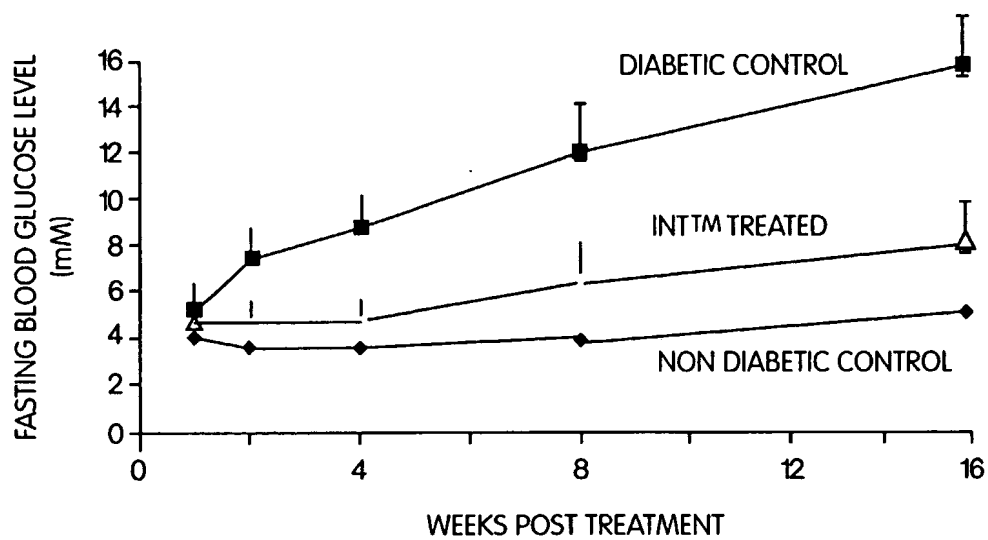


Fig. 6

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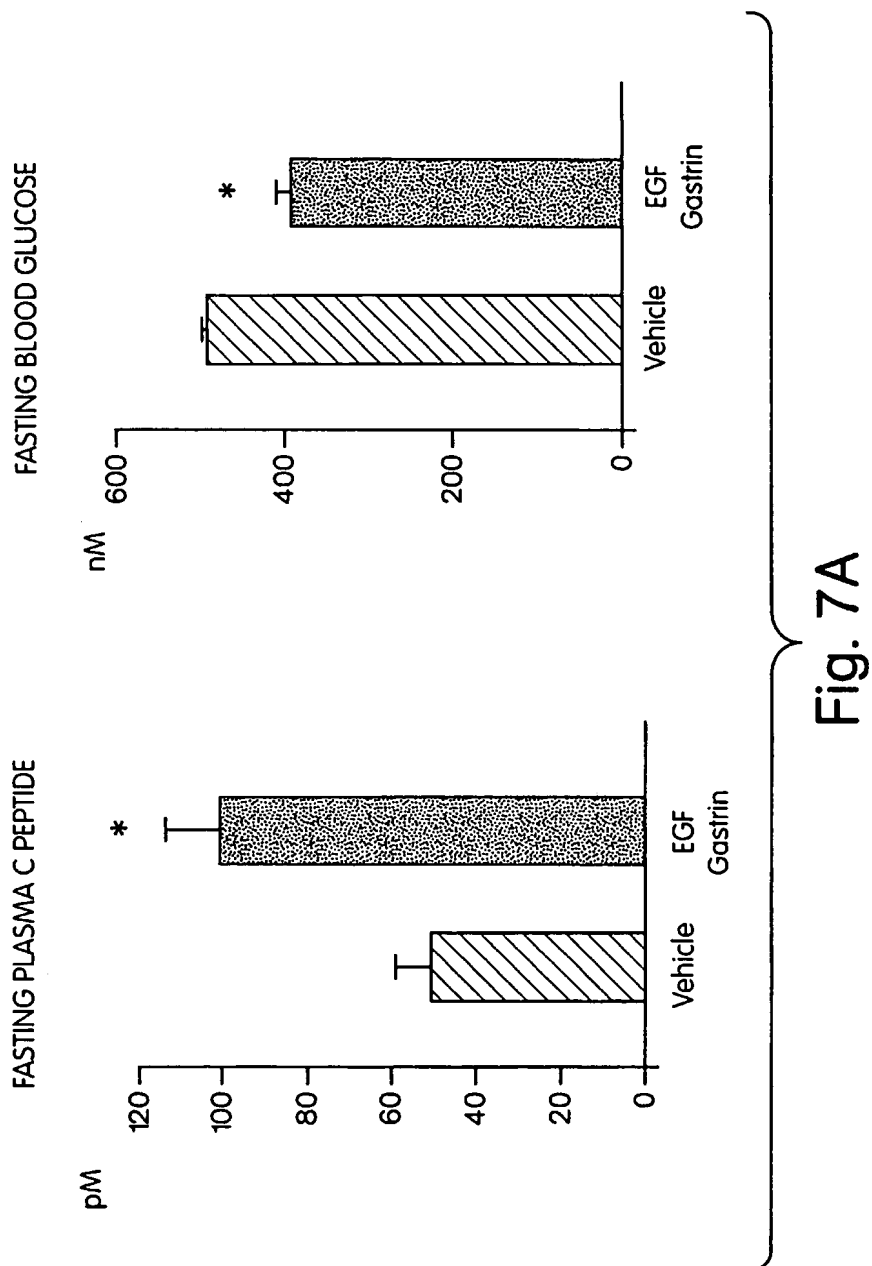


Fig. 7A

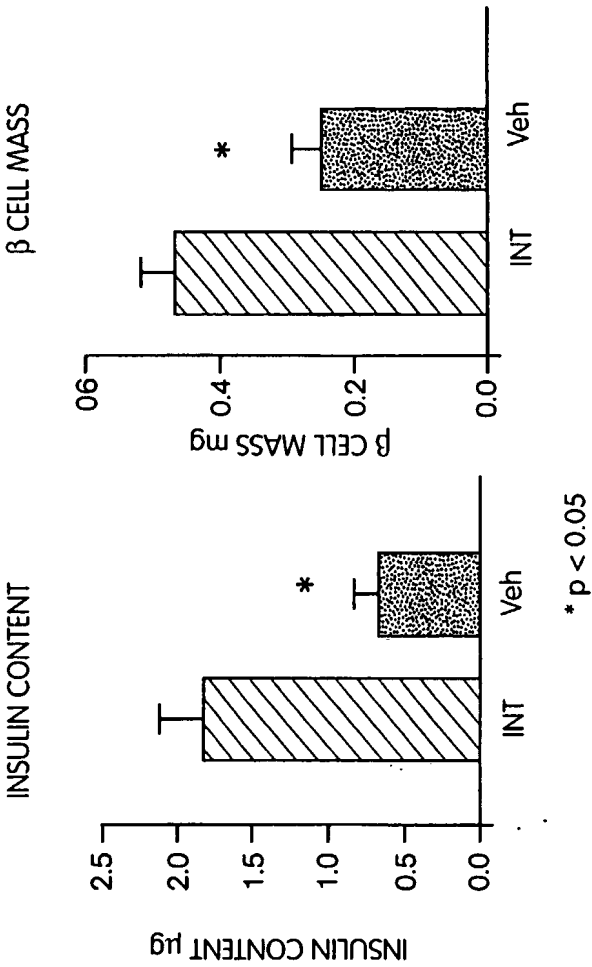


Fig. 7B

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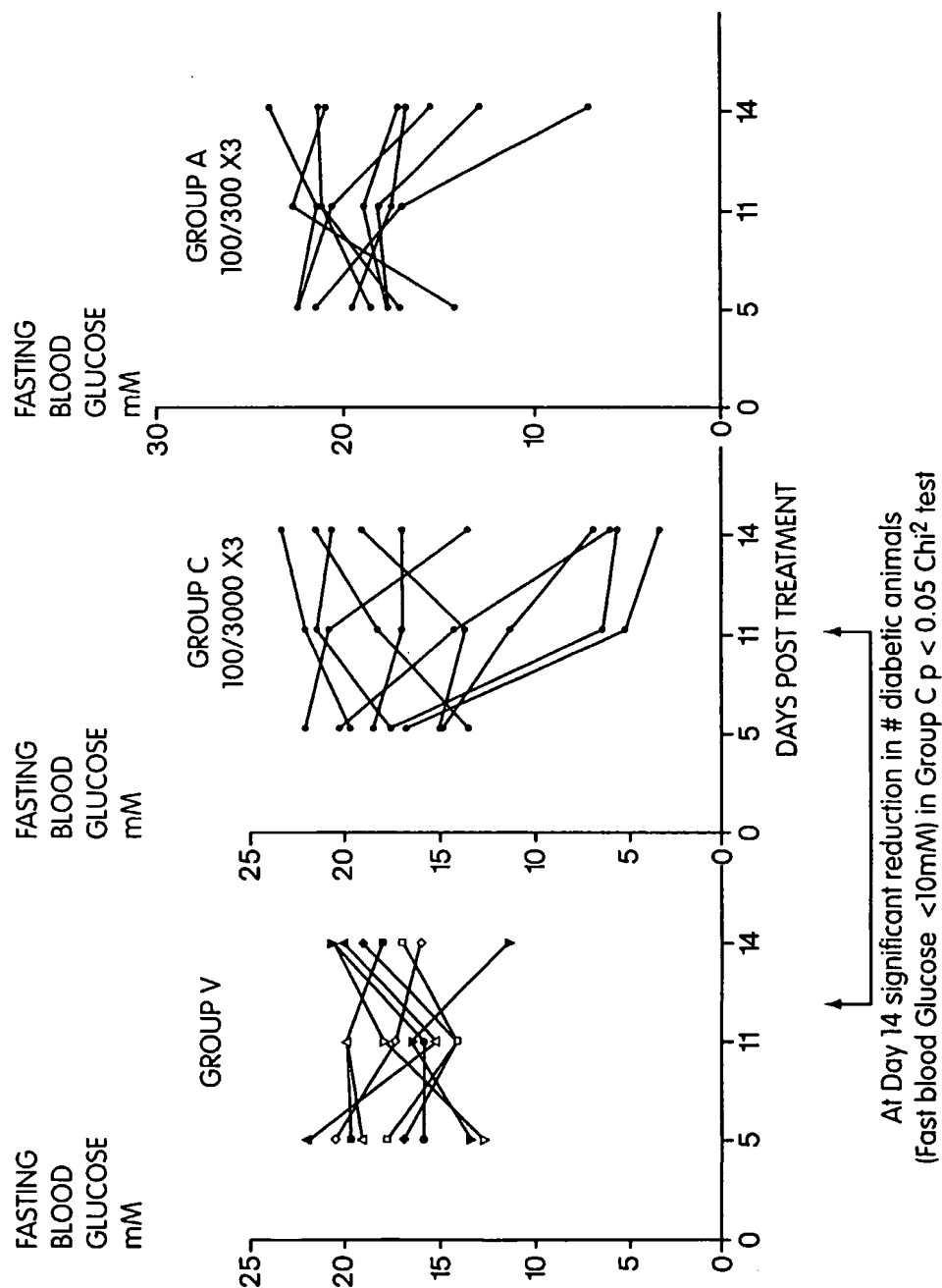


Fig. 8